Species Identification and Variation in the North American Cranberry Fruit Rot Complex

J.J. Polashock[1a], P. Oudemans[2] and C. Constantelos[2]
[1]USDA-ARS and [2]Rutgers University
Blueberry/Cranberry Research Center
Chatsworth, NJ 08019
USA

F.L. Caruso
University of Massachusetts
Cranberry Experiment Station
East Wareham, MA 02538-0569
USA

P. McManus
Dept. of Plant Pathology
University of Wisconsin
Madison, WI 53706
USA

J. Crouch
Rutgers University
Dept. of Plant Biology and Pathology
New Brunswick, NJ 08901
USA

Keywords: *Vaccinium macrocarpon,* phylogenetics, fungal pathogens, rDNA, ITS

Abstract

Many different species of pathogenic fungi cause cranberry fruit rot. The contribution of any given species can be quite variable depending on a host of cultural and environmental factors. Control of fruit rot can be problematic in the Northeast and in other growing regions since losses due to fruit rot can be episodic. One possible cause for inadequate control is that pathogenic fungi typically have differential sensitivity to fungicides. Thus, variation in the species or populations of the fungi present can affect the success and quality of control. Our primary objective in this project was to determine the variation within five species of cranberry fruit rot pathogens (*Phyllosticta vaccinii*, *Coleophoma empetri*, *Colletotrichum acutatum*, *Colletotrichum gloeosporioides*, and *Physalospora vaccinii*) across geographic regions [NJ, MA, WI and British Columbia (BC)] using morphologic and molecular characters. Our secondary objective was to reexamine the taxonomy of these pathogens at the molecular level, particularly those that are cranberry- or *Vaccinium* species-specific. Our results suggest that morphologically, within-species variation was low. We did however collect atypical non-pigmented *C. acutatum* from BC and *Physalospora vaccinii* isolates from all regions varied from dark gray to pure white on V8 juice agar medium. Molecular analysis of ribosomal ITS sequences showed a low level of variability for most pathogen species, with the exception of the morphologically distinct isolates. Phylogenetic analysis showed that the atypical, non-chromogenic *C. acutatum* formed a distinct, but closely related clade, to the normal pink-pigmented isolates. In contrast, the pure white isolates of *Physalospora vaccinii* possessed an ITS sequence distinct from those of the gray strains, suggesting they may represent a different species.

INTRODUCTION

Cranberry fruit rot (CFR) is caused by a complex of fungi that can reduce yields and increase fresh fruit storage losses. The problem affects all North American growing regions, but is particularly problematic in New Jersey and Massachusetts where CFR can destroy 50 to 100% of the fruit if the crop is not protected by fungicides (Oudemans, et al., 1998). The impact of CFR in Wisconsin is variable with certain pathogens being important in limited locations or years, while in the western cranberry growing regions of Washington, Oregon and British Columbia, losses due to fruit rot occur, but not nearly to the same extent as in NJ and MA.

[a] James.Polashock@ARS.USDA.GOV

Even with the routine use of fungicides, there is great variability in the success of fruit rot control. We hypothesized that this could be partially due to variability both in the species prevalent, as well as variation within those species. As a first step toward surveying this variability, we collected rotted cranberry fruit from all major growing regions and recovered representative isolates of *Phyllosticta vaccinii*, *Coleophoma empetri*, *Colletotrichum acutatum*, *Colletotrichum gloeosporioides*, and *Physalospora vaccinii*. We used morphological and molecular approaches to characterize variability and taxonomic relationships.

MATERIALS AND METHODS

Rotted cranberry fruit was collected from several locations in New Jersey, Massachusetts, Wisconsin and British Columbia (BC) in Sept. 2005 and Sept. 2006. Berries were surface sterilized for 5-20 min in 10% bleach solution, rinsed in sterile water, halved crosswise and placed cut side down on nutrient agar plates (V8 juice agar, potato dextrose agar, or corn meal agar). The plates were incubated at room temperature for 5-10 days and fungi growing from the rotted fruit were subcultured to fresh plates. An effort was made to collect about 10 isolates of each of the five pathogen species (*Phyllosticta vaccinii*, *Coleophoma empetri*, *Colletotrichum acutatum*, *Colletotrichum gloeosporioides*, and *Physalospora vaccinii*) from each of the four growing regions. The isolates chosen for further analysis were selected to represent the range of variation within a species. Morphological characters, such as mycelium pigmentation, growth habit, spore size, and spore shape, were recorded for each isolate.

DNA was extracted from all fungal species grown on V8 juice agar for 7-10 days. Approximately 20 mg of aerial mycelium was suspended in 500 µl of CTAB buffer (Stewart and Via, 1993) and frozen at -20 °C for at least 30 min followed by heating at 65 °C for 30 min. The suspension was extracted twice with an equal volume of chloroform and total nucleic acid was precipitated from the supernatant using 0.7 volumes of isopropanol. Pellets were rinsed, resuspended in 1 ml of TE buffer and treated with RNase A at 37 °C for 30 min, followed by chloroform extraction. After a final ethanol precipitation, DNA was resuspended in 25 µl of TE (pH 7.6).

The nuclear internal transcribed spacer (ITS) 1, 5.8 ribosomal RNA gene and ITS 2 region was amplified by PCR using the universal fungal ITS primers ITS1 and ITS4 (White et al., 1990). PCR products were direct sequenced after treatment with ExoSAP-IT (USB Corp., Cleveland, OH) or cloned into a plasmid vector for amplification and sequencing. Sequencing reactions were performed using the DCTS Quick Start kit (Beckman Coulter, Fullerton, CA) and run on a CEQ 8000 (Beckman Coulter). Sequence data was assembled and edited using various modules of the Lasergene Software package (DNAStar Inc., Madison, WI).

Similarity to published sequences was determined by performing BLAST searches (Altschul et al., 1990) of the NCBI GenBank database. Multiple sequence alignments were prepared using the program T-Coffee (Poirot, et al, 2003), followed by manual refinements in the program BioEdit (Hall, 2007). Phylogenetic trees were generated from maximum likelihood (ML) searches executed in PAUP* using heuristic search strategies with 10 random addition sequence replicates and tree-bisection reconnection branch swapping.

RESULTS

Physalospora vaccinii

This species was isolated from all cranberry growing regions except BC. Morphological features of colonies grown in culture were consistent with published descriptions for the species (Shear, et al., 1931). Isolates varied in pigmentation from a dark gray to pure white and some sectoring was evident. Pure white isolates were less frequently isolated, with one collected from the NJ and three from WI. The darker isolates sporulated profusely, while the white isolates sporulated sparsely. Those isolates that

were varying shades of gray all had a similar ascospore size and shape, while those from the pure white isolates were distinct.

Although morphologically the isolates evaluated in this study agreed with the historical description of *Physalospora vaccinii*, BLAST searches using our sequence data suggested that our isolates were more similar to members of the sub-class Sordariomycetidae, while *Physalospora* sp. are reported to be members of the sub-class Xylariomycetidae. In addition, phylogenetic analysis showed that the pure white isolates formed a clade distinct from the gray isolates. Despite this distinction, recombination appeared to occur between these two groups.

Colletotrichum acutatum

This pathogen was isolated from all four cranberry growing regions. Almost all isolates had gray mycelium in culture and produced red pigmentation in the medium. Sporulation was prolific in all isolates as evidenced by masses of orange conidia emerging from acervuli. Conidia were fusiform with sizes consistent with the current taxonomic description (Sutton, 1980, 1992).

In addition to those described above, atypical non-chromogenic isolates were recovered from BC. These isolates were similar to the pigmented ones for all other morphological features. ITS sequence analysis and BLAST searches suggested that all isolates, regardless of pigmentation, were in fact *C. acutatum.* However, phylogenetic analysis showed the non-pigmented isolates formed a distinct, but weakly supported clade.

Colletotrichum gloeosporioides

This species was not found in WI, but representative isolates were recovered from all other growing regions. All *C. gloeosporioides* isolates were uniformly gray and non-chromogenic, with no diffusible pigment production in culture. The teleomorph (*Glomerella cingulata*) was intermittently present. Pale yellow masses of conidia were produced in acervuli. The spores were rounded at the ends and variable in size. Perithecia containing ascospores were sometimes formed on the rotted fruit and in pure culture. All morphological characters, including spore size and shape were within the accepted description of this species (Sutton, 1992). BLAST searches of the ITS region sequence from these isolates confirmed association within the broad circumscription of *C. gloeosporioides* sensu lato. However, phylogenetic analysis placed these isolates closer to *C. kahawae,* a species of *Colletotrichum* pathogenic to coffee.

Phyllosticta vaccinii

This species was common to all growing regions except BC. Colonies were dark green with irregular margins and grew slowly on V8 juice agar. Pycnidia formed densely on the cultured fruit as well as the agar medium. Conidia were obovate with a short gelatinous appendage, consistent with published descriptions (Weidemann, et al., 1982). BLAST searches of GenBank using the ITS sequence confirmed these isolates as members of the genus *Phyllosticta.* Our sequences were most similar to those posted as *Phyllosticta pyrolae*, including a sample isolated from Ericaceous plants in Japan. Similarity was also high to an unpublished isolate of *Phyllosticta vaccinii* from cranberry and several sequences from *Guignardia* species, the teleomorph of *Phyllosticta.* Based upon the ITS tree topology, *Phyllosticta vaccinii* is paraphyletic, as an isolate of *Phyllosticta elongata* was positioned within the group.

Coleophoma empetri

This pathogen was common to all growing regions. Mycelium on V8 juice agar was dark gray to black and older cultures developed white crystals in the medium. Margins were smooth and often lobed. Pycnidia commonly formed on the surface of the fruit and on the edge where fruit contacted the growth medium. However, in the absence of the fruit, pycnidia failed to form. Paraphyses were present among the conidiophores

and the conidia were cylindrical, guttulate, and hyaline. The observed morphological characteristics were within the species circumscription as described (Duan, et al., 2007).

These isolates exhibited little sequence variation in the ITS region. BLAST searches showed our cranberry isolates as being similar to a cranberry isolate posted as *Gloeosporium* sp. (EF672242) and to several endophytes and pathogens of the Leotiomycete/Helotiale/Helotiaceae group, consistent with the broader taxonomic placement of *Coleophoma* species. The only sequence of *Coleophoma* in the database (EF672243) was also isolated from cranberry. However, none of our isolates had sequence data similar to that sample. Since that sequence (EF672243) did not possess significant ITS sequence similarity to any member of the Leotiomycete/Helotiale/Helotiaceae group, it is possible that the isolate was incorrectly classified.

DISCUSSION

Although widespread, some species of the pathogens used in this study were not found in all growing regions. For example, *Physalospora vaccinii* was not found in BC. These species could be limited to certain geographic regions or alternatively be present, but at low or variable levels such that they escaped isolation when the fruit for this study was collected. The cranberry industry is over 100 years old and beds can remain in production for decades. In addition, cranberry is typically propagated asexually through cuttings and shipped within and between growing regions to start new beds. Thus it is not surprising that most of the pathogens we studied were as described on cranberry many years ago (Shear, et al., 1931). Regional differences in ITS sequence were generally low. This too could be due to concomitant transport of the pathogens with vines from region to region, resulting in homogenization of the pathogen populations. The idea that all growing regions face many of the same pathogens and those pathogens exhibit less than expected regional diversity is good news from the standpoint of obtaining adequate fruit rot control. Furthermore, the lack of diversity in the ITS regions will allow the development of probes for rapid detection that will be applicable across all growing regions.

Interestingly, the specificity of the fungi for cranberry did not seem to affect the level of diversity. For example, *C. acutatum* is a world wide pathogen affecting many crop species, while *Physalospora vaccinii* is specific to cranberry, yet both species were similarly homogenous at the ITS sequence level. Mutation was however evident in that non-chromogenic *C. acutatum* were recovered from BC. More comprehensive sequence analysis is likely to show more inter-population diversity.

Our *C. gloeosporioides* isolates were more closely related to isolates of *C. kahawae* than the neotype for this species. This suggests a reexamination of this species' taxonomic status is warranted. Similarly, *Phyllosticta vaccinii* sequence data showed a close association with *Phyllosticta pyrolae* and was less similar to an isolate of *Phyllosticta vaccinii* posted in Genbank. One possibility is that this species may be more diverse than originally thought or more than one species in the genus is found on cranberry. *Physalospora vaccinii* also requires reevaluation as our analysis suggests the cranberry isolates are related to a different subclass than other *Physalospora* sp. The isolates commonly identified as *Physalospora vaccinii* from cranberry may in fact be members of a different genus. Future efforts will be directed at addressing these taxonomic questions.

ACKNOWLEDGEMENTS

This work was primarily funded through a USDA-CSREES, PMAP (Pest Management Alternatives Program) grant. We thank Kristy Adams for technical assistance.

Literature Cited

Altschul, S.F., Gish, W., Miller, W., Myers, E.W. and Lipman, D.J. 1990. Basic local alignment search tool. J. Mol. Biol. 215:403-410.

Duan, J., Liu, X. and Wu, W. 2007. Reinstatement of *Coleonema* for *Coleophoma oleae* and notes on *Coleophoma*. Fungal Diversity 26:187-204.

Hall, T. 2007. BioEdit v7.0.9. Biological sequence alignment editor. Distributed by the author at http://www.mbio.ncsu.edu/BioEdit/bioedit.html.

Oudemans, P.V., Caruso, F.L. and Stretch, A.W. 1998. Cranberry fruit rot in the Northeast: A complex disease. Plant Disease 82:1176-1184.

Poirot, O., O'Toole, E. and Notredame, C. 2003. Tcoffee@igs: A web server for computing, evaluating and combining multiple sequence alignments. Nucleic Acids Research 31:3503-3506.

Shear, C.L., Stevens, N.E. and Bain, H.F. 1931. Fungous diseases of the cultivated cranberry. U.S. Dep. Agric. Tech. Bull. 258:58 pp.

Stewart, C.N. and Via, L.E. 1993. A rapid CTAB DNA isolation technique useful for RAPD fingerprinting and other PCR applications. Biotechniques 5 (14):748-750.

Sutton, B.C. 1980. The coelomycetes: fungi imperfecti with pycnidia, acervuli, and stromata. Kew, U.K.: Commonwealth Mycological Institute.

Sutton, B.C. 1992. *The genus Glomerella and its anamorph Colletotrichum*. Edited by J. A. Bailey and M. J. Jeger, In Colletotrichum: Biology, pathology and control. Wallingford, U.K.: CAB International.

Weidemann, G.J., Boone, D.M. and Burdsall, H.H. 1982. Taxonomy of *Phyllosticta vaccinii* (Coleomycetes) and a new name for the true anamorph of *Botryosphaeria vaccinii* (Dothidiales, Dothioraceae). Mycologia 74:59-65.

White, T.J., Burns, T., Lee, S., and Taylor, J. 1990. Amplification and direct sequencing of fungal ribsomal RNA genes for phylogenetics. In PCR Protocols, edited by M.A. Innis, D.H. Gelfand, J.J. Sninsky and T. J. White. New York: Academic Press Inc.